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Kinetics of long-term degradation of different molar mass hyaluronan solutions studied by SEC-MALLS

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ABSTRACT

The degradation of hyaluronan of four molecular weights in aqueous solutions containing sodium azide as a protectant was studied by SEC-MALLS determination of molecular mass and polydispersity and conformation parameters. The solutions were stored either at laboratory or refrigerator temperatures for up to seven months. After this time the molar mass decreased in 9–15% (room temperature) or 5–10% (fridge) depending also on the storage conditions of solid samples from which the solutions were prepared. Two degradation phases were observed at room temperature conditions. Two kinetic models were employed to fit the data – zero order and exponential with no statistical preferences of any of them. The polydispersity of all samples was low and remained unaltered during the whole degradation at both temperatures which indicates non-random mechanism of degradation.

1. Introduction

One of the most important polysaccharide originated in mammal body is sodium salt of hyaluronic acid (HA). Its importance strongly increased in the last two decades. A lot of studies have focused on its physical-chemical behaviour, role in tissue, cell proliferation and developed ways how to use this polysaccharide in wound healing, drug delivery system, anti-ageing application and so on.

Hyaluronan is a linear natural polysaccharide of the glycosaminoglycans family. Its chemical structure comprises disaccharide units composed of D-glucuronic acid and N-acetyl-D-glucosamine, which are alternatively linked through 1,3 and 1,4 glycosidic bonds [1].

Different molar mass hyaluronan has different role in body. For example high molar mass hyaluronan organizes extracellular matrix and low molar mass hyaluronan can be found in injured tissue or in certain tumours [2,3]. This means, that high molar mass hyaluronan in human body reflects normal tissue, but low molar mass hyaluronan reflects damaged or stressed tissue [4].

For the above mentioned reasons many scientific groups reported studies how to influence hyaluronan molar mass or what can cause its degradation. Most of methods how to cleave hyaluronan is summarized in review from Stern et al. published in 2007 [4]. They describe enzymatic degradation and non-enzymatic degradation of hyaluronan. Enzymatic degradation seems to be most suitable for preparation of hyaluronan fragments with lower molar mass. One of the important reasons is scission of the glycosidic linkages exclusively without modification of primary structure of hyaluronan.

Low or high pH conditions have obvious effect on hyaluronan. Hydrolysis occurs in acid solution on the glucuronic acid residue and the hemiacetal ring remains. In basic solution, hydrolysis occurs on N-acetylglucosamine residue. The hydrolysis obeys first order kinetics [5]. Other important observation suggested that random chain scission occurs during hydrolytic degradation [1,5,6]. As well as random chain scission occurs during hydrolytic degradation, the same mechanism was proposed for thermal degradation of hyaluronan [7,8]. The opposite conclusion to hydrolysis and thermal degradation was made to ultrasonication. Hyaluronan degrades in a non-random way when exposed to ultrasound resulting in a bimodal molar mass distribution [9]. Ultrasonication is preferable to other applied degradative procedures (i.e. conventional heating and microwave irradiation), because ultrasonication can reduce molar mass up to 100 kDa without significant changes in primary structure of hyaluronan [10].

Other possible ways of hyaluronan degradation can be presented, such as interaction with hydrogen peroxide [11], ozone [12], singlet oxygen and so on, but it would be beyond the scope of this paper and all of these methods are summarized in the review by

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Stern et al. [4] or some of these aspects are also in the review from Lapčík et al. [1]. Hyaluronan is a strongly hydrophilic molecule [13].

There is very little information on the stability of hyaluronan molecular mass in solutions after hyaluronan powder is dissolved in water. In other words, little information on degradation of hyaluronan solutions prepared and stored in laboratory for various purposes. This is the first report on this issue.

2. Materials and methods

The degradation of hyaluronan samples of four different molar masses was studied by SEC-MALLS technique in order to observe the changes of molar mass, polydispersity and polymer conformation in time. All hyaluronic acid samples which were at disposal for this study were obtained from Contipro (Czech Republic) where were produced by fermentation (Streptococcus equi., subsp. Zooepidemicus bacterial strain). The product names and their actual weight averaged molar masses, given by the producer who had measured it also with SEC-MALLS when the fresh solid powder of hyaluronan was obtained by fermentation, were as follows:

- HA 1 MDa: $M_w = 1$ MDa;
- HA 0.75 MDa: $M_w = 752$ kDa;
- HA 200–300 kDa: $M_w = 267$ kDa;
- HA 10–150 kDa: $M_w = 17$ kDa.

These solid materials were kept in different conditions before used for this study and were thus subjected to some potential degradation in the solid state:

- HA 1 MDa: 6 years old, kept in the fridge (5–7 °C) during this period;
- HA 0.75 MDa: one year old, kept in fridge during this period;
- HA 200–300 kDa: one year old, kept in desiccator at room temperature during this period;
- HA 10–150 kDa: the powder was obtained just few weeks before this study and kept in fridge prior to analysis.

Thus also some information on the degradation during the storage in the solid state could be obtained besides the main goal – study of degradation in solution.

The SEC-MALLS equipment used in the present work was produced by Wyatt (USA; the detector part) and by Agilent (USA; the chromatography part). It included MALLS detector (Dawn Heleos II), viscometric detector (ViscoStar II) and RI detector (Optilab T-rEX). The multi angle laser light scattering detector had 18 angles of detection, ranging from 10° to 160°. Astra 6 software package was used for data collection and analysis.

The chromatograph contained degasser, isocratic pump, autosampler, column for size exclusion chromatography (one PL aquagel-OH MIXED-H 8 μm PL1149-6800 produced by Agilent was used), and thermostat. The mobile phase used was 0.1 M $NaNO_3$ aqueous solution, containing 3 mM NaN_3 to prevent microorganism growing. Sodium azide is commonly used protectant of hyaluronan solutions. All SEC-MALLS measurements were performed at 25 °C.

The solutions of hyaluronic acid were prepared with the same solvent as the mobile phase. We used solutions of different concentrations, ranging from 1 mg/ml for high molar mass samples hyaluronan up to 5 mg/ml for low molar mass samples.

SEC-MALLS technique allows:

- the separation of different polymeric compounds (fractions) according to their molar masses
- the determination of absolute molar mass averages from 10^2 Da to 10^9 Da
- the calculation of polydispersity
- the determination of the root mean square radius (RMS), also known as "radius of gyration" (R_g)
- the determination of conformation plot and Mark-Houwink-Sakurada (MHS) plot

From the slope of the conformation plot RMS = $f(M_w)$ the shape of the polymer can be found [14–16]. The shape of the polymer can be obtained as well from Mark-Houwink-Sakurada plot [14,17–20].

For molar mass calculation Zimm model was used [21,22]. The refractive index increment (dn/dc) value used to calculate the molar mass was 0.165 mL/g [23,24].

3. Results and discussions

As noted in the preceding section, the degradation of hyaluronan samples could occur first in the solid state during the storage of the powdered samples before this study and then occurred in aqueous solutions prepared for this study. The degradation in the solid state really occurred, except for HA 10–150 kDa (the shortest storage in the solid state), as was confirmed by the SEC-MALLS analysis of the fresh prepared solutions which served as initial points for this study (Table 1).

The degradation in the solid state expressed as the molar mass loss relatively to the value given by the producer was as follows: 47.4% for HA 1 MDa, 15.8% for HA 0.75 MDa, 34.2% for HA 200–300 kDa. The degradation corresponds to the storage conditions as expected – higher molar mass loss is observed for the longer storage and/or for the storage at higher temperature. From these values an overall degradation rate of solid preparations could be estimated in %/year: 7.9 for HA 1 MDa, 15.8 for HA 0.75 MDa, 34.2 for HA 200–300 kDa. We will come back to degradation kinetics below but it is clear that the degradation is faster during the storage at laboratory temperature.

As an example, the SEC-MALLS record obtained for the fresh solution of HA 10–150 kDa sample is shown in Fig. 1 (in this case concentration was 2.5 mg/ml). The standard deviation of the results obtained with SEC-MALLS for the same sample at the same conditions was around 1%.

After measuring the fresh solutions, an amount of the prepared solutions was kept at room temperature, and other amount was kept in the fridge. The solutions were then analyzed after one week, one month, 3, 5 and 7 months (the low molar mass samples were also measured at one month and a half). In Table S1(supplementary information) are the values of weight average molar mass (M_w) and mass loss for all solutions measured at different periods.

At room temperature, we observed an exponential or perhaps hyperbolic decrease of M_w in time (Figs. 2 and 3). We can see that after certain period of fast decrease of molar mass, the degradation rate at room temperature is very small.

After more than 90 days in the fridge, only a small decrease in molar mass was observed, especially for the samples with higher molar masses. The values of molar mass found after 90 days for the samples kept in the fridge were still higher than the values of molar

Table 1
Weight averaged molar mass (M_w) and polydispersity determined for fresh hyaluronan solutions.

Sample name	M_w kDa	Polydispersity
HA 1 MDa	526	1.20
HA 0.75 MDa	633	1.30
HA 200–300 kDa	176	1.25
HA 10–150 kDa	17.1	1.10